NUCLEIC ACID DETECTION ASSAY CONTROL GENES

INVENTORS: ARTHUR CASTLE, BRANDON HIGGS, MICHAEL ELASHOFF AND MARK PORTER

FIELD OF THE INVENTION

[0001] The invention relates generally to control genes that may be utilized for normalizing hybridization and/or amplification reactions, as well as methods of identifying these genes that may be used in toxicology studies and in analyzing gene expression data sets for quality and compatibility with other data sets.

RELATED APPLICATIONS

[0002] This application claims priority under 35 U.S.C. §119(e) to U.S. Provisional Application 60/396,145, filed July 17, 2002, which is herein incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

[0003] Nucleic acid hybridization and other quantitative nucleic acid detection assays are routinely used in medical and biotechnological research and development, diagnostic testing, drug development and forensics. Such technologies have been used to identify genes which are up- or down-regulated in various disease or physiological states, to analyze the roles of the members of cellular signaling cascades and to identify drugable targets for various disease and pathology states.

[0004] Examples of technologies commonly used for the detection and/or quantification of nucleic acids include Northern blotting (Krumlauf (1994), *Mol Biotechnol* 2:227-242), *in situ* hybridization (Parker & Barnes (1999), *Methods Mol Biol* 106:247-283), RNAse protection assays (Hod (1992), *Biotechniques* 13:852-854; Saccomanno *et al.* (1992), *Biotechniques* 13:846-850), microarrays, and reverse transcription polymerase chain reaction (RT-PCR) (see Bustin (2000), *J Mol Endocrin* 25:169-193).

[0005] The reliability of these nucleic acid detection methods depend on the availability of accurate means for accounting for variations between analyses. For example, variations in hybridization conditions, label intensity, reading and detector efficiency, sample

concentration and quality, background effects, and image processing effects each contribute to signal heterogeneity (Hegde *et al.* (2000), *Biotechniques* 29:548-562; Berger *et al.* (2000), WO 00/04188). Normalization procedures used to overcome these variations often rely on control hybridizations to housekeeping genes such as β-actin, glyceraldehyde-3-phosphate dehydrogenase (GADPH), and the transferrin receptor gene (Eickhoff *et al.* (1999), *Nuc Acids Res* 27:e33; Spiess *et al.* (1999), *Biotechniques* 26: 46-50). These methods, however, generally do not provide the signal linearity sufficient to detect small but significant changes in transcription or gene expression (Spiess *et al.* (1999), *Biotechniques* 26: 46-50). In addition, the steady state levels of many housekeeping genes are susceptible to alterations in expression levels that are dependent on cell differentiation, nutritional state, specific experimental and stimulation protocols (Eickhoff *et al.* (1999), *Nuc Acids Res* 27:e33; Spiess *et al.* (1999), *Biotechniques* 26:46-50; Hegde *et al.* (2000), *Biotechniques* 29:548-562; and Berger *et al.* (2000), WO 00/04188). Consequently, there exists a need for the identification and use of additional genes that may serve as effective controls in nucleic acid detection assays.

SUMMARY OF THE INVENTION

[0006] The present invention includes methods of identifying at least one gene that is consistently or invariantly expressed across different cell or tissue types in an organism, comprising: preparing gene expression profiles for different cell or tissue types from the organism; calculating a percent variability of expression for at least one gene in each of the profiles across the different cell or tissue types; and selecting any gene whose percent variability of expression indicates that the gene is consistently or invariantly expressed across the different cell or tissue types. The percent variability of expression may be determined by a one-factor or two-factor analysis of variance (ANOVA) wherein the R^2 value is a measure of percent variability of expression.

[0007] The invention, in another embodiment, includes methods of normalizing the data from a nucleic acid detection assay comprising: detecting the expression level for at least one gene in a nucleic acid sample; and normalizing the expression of said at least one gene with the detected expression of at least one control gene of Table 1. The number of control genes used to normalize gene expression data may comprise about 10, 25, 50, 100, 500 or more of the control genes herein identified.

[0008] In another embodiment, the invention includes a set of probes comprising at least two probes that specifically hybridize to a gene of Table 1. The set may comprise at least about 10, 25, 50, 100, 500 or more of the control genes of Table 1. The sets of probes may or may not be attached to a solid substrate such as a chip.

DETAILED DESCRIPTION

[0009] The present Inventors have identified rat control genes that may be monitored in nucleic acid detection assays and whose expression levels may be used to normalize gene expression data or evaluate the suitability of test data to compare to or to include in a database of like data. Normalization of gene expression data from a cell or tissue sample with the expression level(s) of the identified control genes allows the accurate assessment of the expression level(s) for genes that are differentially regulated between samples, tissues, treatment conditions, *etc.* These control genes may be used across a broad spectrum of assay formats, but are particularly useful in microarray or hybridization based assay formats.

A. Nucleic Acid Detection Assay Controls

1. Selection of Control Genes

[0010] As used herein, the genes selected by the disclosed methods as well as the rat genes and nucleic acids of Table 1 (identified by ANOVA methods, discussed below) are referred to as "invariant" or "control genes." Control genes of the invention may be produced by a method comprising preparing gene expression profiles (a representation of the expression level for at least one gene, preferably 10, 25, 50, 100, 500 or more, or, most preferably, nearly all or all expressed genes in a sample) from at least two (or a variety) of cell or tissue types, or from a set of samples of at least one cell or tissue type in which the set contains normal samples (from healthy animals), disease state samples, toxin-exposed samples, etc., measuring the level of expression for at least one gene in each of the gene expression profiles to produce gene expression data, calculating the variation in expression level (R^2) from the gene expression data for each gene and selecting genes whose variation in expression level indicates that the gene is consistently expressed at about the same level in the different cell or tissue types. In one embodiment, such genes that are expressed at

-4-

about the same level, or are invariantly expressed, are those genes that have a percent variability in expression level (R^2) less than or equal to about 12.

[0011] In preferred embodiments, the statistical measure referred to herein as the percent variability in expression level (R^2) is calculated on a gene by gene basis across a number of samples or across a reference database to find the least variant genes with respect to a number of cell or tissue types or sample treatments. A two-factor ANOVA model is applied to all cell and tissue sample sets where both control and disease, pathology or treatment groups exist. The factors for this model were normal state (control or affected tissue) and tissue type. A one factor ANOVA was also used to examine the effects of tissue kind alone. Genes are ranked according to R-squared values. The R-squared value can be interpreted as the percent variability of expression that can be explained by the underlying factors. Cut-off values are also selected for the alpha error p-values for each factor and the interaction of these two factors. A cut-off value for both one factor and two factor R^2 values of less than or equal to about 14, preferably less than about 12, may be used, and genes with R^2 values less than or equal to 14, preferably less than or equal to 12, may be selected as control genes or considered as genes that are consistently expressed across the different cell or tissue types tested. In addition, any gene with large known regulation events within tissues may be removed and any co-clustered Unigene fragments may be examined for consistency in R^2 values. A probe set is also selected using the following supplemental criteria: (a) Mean Average Differential over all rat samples less than or equal to about 20, (b) Present Frequency over all rat samples less than or equal to about 75% and (c) no probe sets exhibiting saturation.

Model 1: $E_{ij} = u + T_j + error$

(E$_{ij}$ is the expression value of the ith gene in the jth sample)

(T$_j$ is the tissue type of the jth sample)

[0012] For each gene, model fitting produces a p-value for the T factor, as well as a sum of squares attributable to this factor. This sum of squares is the model sum of squares. The R^2 value is then the ratio of the model sum of squares to the total sum of squares $\sum_j (E_{ij} - \bar{E}_i)^2$.

Model 2: $E_{ij} = u + T_j + N_j + T_j*N_j + error$

(E$_{ij}$ is the expression value of the ith gene in the jth sample)

(T_j is the tissue type of the jth sample)

(N_j is the state of the jth sample ($N_j = 0$ for normal, 1 otherwise))

[0013]   The model fitting yields, for each gene, a p-value for the T factor, the N factor, and the T*N factor, as well as a sum of squares attributable to each of these factors. Adding the three sums of squares gives the model sum of squares. The R^2 value is then the ratio of the model sum of squares to the total sum of squares

$$\sum_j (E_{ij} - \bar{E}_i)^2.$$

[0014]   Further, the ANOVA-based methods of the invention are particularly useful for determining the compatibility of a test sample to an entire set of samples, or an existing database derived from those samples. For instance, an R^2 value for genes that have been shown to be the most resistant to variability is calculated for all samples within a test group or test database. These R^2 values are then compared to those from a standard reference database. Accordingly, a closeness distribution of all individual samples in the test database to the reference database as a whole can be generated to evaluate the compatibility of new samples. The genes identified in Table 1 show invariant patterns of expression and can be used to assess compatibility and reliability of gene expression experiments and predictive modeling experiments. These genes show low variability both in control groups from many different experiments and in studies of disruptions of gene expression, such as those occurring in disease states. As a result, these genes can be used as an internal standard for comparing gene expression data. Measurements of expression level of these genes are used to determine the extent of compatibility of data from different sources and the need, or lack thereof, for normalization or further quality control and adjustments. These measurements also provide an internal standard that supplies a reference point for highly disrupted patterns of gene expression. These genes are also of critical importance for determining relative expression if small numbers of markers are used in custom microarrays.

[0015]   In some embodiments of the invention, the percent variability of expression may be calculated from data that has been normalized to control for the mechanics of hybridization, such as data normalized or controlled for background noise due to non-specific hybridization. Such data typically include, but are not limited to, fluorescence readings from microarray based hybridizations, densitometry readings produced from

assays that rely on radiological labels to detect and quantify gene expression and data produced from quantitative or semi-quantitative amplification assays.

[0016] In the methods of the invention, gene expression profiles may be produced by any means of quantifying gene expression for at least one gene in the tissue or cell sample. In preferred methods, gene expression is quantified by a method selected from the group consisting of a hybridization assay or an amplification assay. Hybridization assays may be any assay format that relies on the hybridization of a probe or primer to a nucleic acid molecule in the sample. Such formats include, but are not limited to, differential display formats and microarray hybridization, including microarrays produced in chip format. Amplification assays include, but are not limited to, quantitative PCR, semiquantitative PCR and assays that rely on amplification of nucleic acids subsequent to the hybridization of the nucleic acid to a probe or primer. Such assays include the amplification of nucleic acid molecules from a sample that are bound to a microarray or chip.

[0017] In other circumstances, gene expression profiles may be produced by querying a gene expression database comprising expression results for genes from various cell or tissue samples. The gene expression results in the database may be produced by any available method, such as differential display methods and microarray-based hybridization methods. The gene expression profile is typically produced by the step of querying the database with the identity of a specific cell or tissue type for the genes that are expressed in the cell or tissue type and/or the genes that are differentially regulated compared to a control cell or tissue sample. Available databases include, but are not limited to, the Gene Logic ToxExpress® database, the Gene Expression Omnibus gene expression and hybridization array repository available through NCBI (www.ncbi.nlm.nih.gov/entrez) and the SAGE™ gene expression database.

[0018] The cell or tissue samples that are used to prepare gene expression profiles may include any cell or tissue sample available. Such samples include, but are not limited to, tissues removed as surgical samples, diseased or normal tissues, *in vitro* or *in vivo* grown cells, and cell cultures and cells or tissues from animals exposed to an agent such as a toxin. The number of samples that may be used to calculate absolute R^2 values is variable, but may include about 3, 10, 25, 50, 100, 200, 500 or more cell or tissue samples. The cell or tissue samples may be derived from an animal or plant, preferably a mammal, most preferably a rat. In some instances, the cell or tissue samples may be human, canine (dog), or mouse in origin.

[0019] As used herein, "background" refers to signals associated with non-specific binding (cross-hybridization). In addition to cross-hybridization, background may also be produced by intrinsic fluorescence of the hybridization format components themselves.

[0020] "Bind(s) substantially" refers to complementary hybridization between an oligonucleotide probe and a nucleic acid sample and embraces minor mismatches that can be accommodated by reducing the stringency of the hybridization media to achieve the desired detection of the nucleic acid sample.

[0021] The phrase "hybridizing specifically to" refers to the binding, duplexing or hybridizing of a molecule substantially to or only to a particular nucleotide sequence or sequences under stringent conditions when that sequence is present in a complex mixture (*e.g.*, total cellular) DNA or RNA.

2.   *Preparation of Controls Genes, Probes and Primers*

[0022] The control genes listed in Table 1 may be obtained from a variety of natural sources such as organisms, organs, tissues and cells. The sequences of known genes are in the public databases. The GenBank Accession Number corresponding to the Normalization Control Genes can be found in Table 1. The sequences of the genes in GenBank (http://www.ncbi.nlm.nih.gov/) are herein incorporated by reference in their entirety as of the priority date of this application.

[0023] Probes or primers for the nucleic acid detection assays described herein that specifically hybridize to a control gene may be produced by any available means. For instance, probe sequences may be prepared by cleaving DNA molecules produced by standard procedures with commercially available restriction endonucleases or other cleaving agents. Following isolation and purification, these resultant normalization control gene fragments can be used directly, amplified by PCR methods or amplified by replication or expression from a vector.

[0024] Control genes and control gene probes or primers (*i.e.*, synthetic oligonucleotides and polynucleotides) are most easily synthesized by chemical techniques, for example, the phosphoramidite method of Matteucci, *et al.* ((1981) *J Am Chem Soc* 103:3185-3191) or using automated synthesis methods using the GenBank sequences disclosed in Table 1. Probes for attachment to microarrays or for use as primers in amplification assays may be produced from the sequences of the genes identified herein using any available software,

including, for instance, software available from Molecular Biology Insights, Olympus Optical Co. and Premier Biosoft International.

[0025] In addition, larger nucleic acids can readily be prepared by well known methods, such as synthesis of a group of oligonucleotides that define various modular segments of the normalization control genes and normalization control gene segments, followed by ligation of oligonucleotides to build the complete nucleic acid molecule.

B. Normalization Methods

[0026] Gene expression data produced from the control genes in a given sample or samples may be used to normalize the gene expression data from other genes using any available arithmatic or calculative means. In particular, gene expression data from the control genes in Table 1 are useful to normalize gene expression data for toxicology testing or modeling in an animal model, preferably in a rat. Such methods include, but are not limited, methods of data analysis described by Hegde *et al.* (2000), *Biotechniques* 29: 548-562; Winzeller *et al.* (1999), *Meth Enzymol* 306:3-18; Tkatchenko *et al.* (2000), *Biochimica et Biophysica Acta* 1500:17-30; Berger *et al.* (2000), WO 00/04188; Schuchhardt *et al.* (2000), *Nuc Acids Res* 28:e47; Eickhoff *et al.* (1999), *Nuc Acids Res* 27:e33. Micro-array data analysis and image processing software packages and protocols, including normalization methods, are also available from BioDiscovery (http://www. biodiscovery.com), Silicon Graphics (http://www.sigenetics.com), Spotfire (http://www. spotfire.com), Stanford University (http://rana.Stanford.EDU/software), National Human Genome Research Institute (http://www.nhgri.nih.gov/DIR/LCG/15K/HTML/ img_analysis .html), TIGR (http://www.tigr.org/softlab), and Affymetrix (affy and maffy packages), among others.

C. Assay or Hybridization Formats

[0027] The control genes of the present invention may be used in any nucleic acid detection assay format, including solution-based and solid support-based assay formats. As used herein, "hybridization assay format(s)" refer to the organization of the oligonucleotide probes relative to the nucleic acid sample. The hybridization assay formats that may be used with the control genes and methods of the present invention include assays where the nucleic acid sample is labeled with one or more detectable labels,

assays where the probes are labeled with one or more detectable labels, and assays where the sample or the probes are immobilized. Hybridization assay formats include but are not limited to: Northern blots, Southern blots, dot blots, solution-based assays, branched-DNA assays, PCR, RT-PCR, quantitative or semi-quantitative RT-PCR, microarrays and biochips.

[0028] As used herein, "nucleic acid hybridization" simply involves contacting a probe and nucleic acid sample under conditions where the probe and its complementary target can form stable hybrid duplexes through complementary base pairing (see Lockhart *et al.*, (1999) WO 99/32660). The nucleic acids that do not form hybrid duplexes are then washed away leaving the hybridized nucleic acids to be detected, typically through detection of an attached detectable label.

[0029] It is generally recognized that nucleic acids are denatured by increasing the temperature or decreasing the salt concentration of the buffer containing the nucleic acids. Under low stringency conditions (*e.g.*, low temperature and/or high salt) hybrid duplexes (*e.g.*, DNA-DNA, RNA-RNA or RNA-DNA) will form even where the annealed sequences are not perfectly complementary. Thus, specificity of hybridization is reduced at lower stringency. Conversely, at higher stringency (*e.g.*, higher temperature or lower salt) successful hybridization requires fewer mismatches. One of skill in the art will appreciate that hybridization conditions may be selected to provide any degree of stringency. In a preferred embodiment, hybridization is performed at low stringency, in this case in 6× SSPE-T at 37°C (0.005% Triton x-100) to ensure hybridization, and then subsequent washes are performed at higher stringency (*e.g.*, 1× SSPE-T at 37°C) to eliminate mismatched hybrid duplexes. Successive washes may be performed at increasingly higher stringency (*e.g.*, down to as low as 0.25× SSPET at 37°C to 50°C until a desired level of hybridization specificity is obtained. Stringency can also be increased by addition of agents such as formamide. Hybridization specificity may be evaluated by comparison of hybridization to the test probes with hybridization to the various controls that can be present (*e.g.*, expression level control, normalization control, mismatch controls, *etc.*).

[0030] As used herein, the term "stringent conditions" refers to conditions under which a probe will hybridize to a complementary control nucleic acid, but with only insubstantial hybridization to other sequences. Stringent conditions are sequence-dependent and will be

different under different circumstances. Longer sequences hybridize specifically at higher temperatures. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH.

[0031] Typically, stringent conditions will be those in which the salt concentration is at least about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide.

[0032] In general, there is a tradeoff between hybridization specificity (stringency) and signal intensity. Thus, in a preferred embodiment, the wash is performed at the highest stringency that produces consistent results and that provides a signal intensity greater than approximately 10% of the background intensity. Thus, in a preferred embodiment, the hybridized array may be washed at successively higher stringency solutions and read between each wash. Analysis of the data sets thus produced will reveal a wash stringency above that the hybridization pattern is not appreciably altered and which provides adequate signal for the particular oligonucleotide probes of interest.

[0033] The "percentage of sequence identity" or "sequence identity" is determined by comparing two optimally aligned sequences or subsequences over a comparison window or span, wherein the portion of the polynucleotide sequence in the comparison window may optionally comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical residue (*e.g.*, nucleic acid base or amino acid residue) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Percentage sequence identity when calculated using the programs GAP or BESTFIT (see below) is calculated using default gap weights. Sequences corresponding to the control genes of Table 1 may comprise at least about 70% sequence identity to the GenBank IDs of the genes in the Tables, preferably about 75%, 80% or 85% or more preferably, about 90% or 95% or more identity.

[0034] Homology or identity is determined by **BLAST** (**B**asic **L**ocal **A**lignment **S**earch **T**ool) analysis using the algorithm employed by the programs blastp, blastn, blastx, tblastn

-11-

and tblastx (Karlin *et al.* (1990), *Proc Natl Acad Sci USA* 87:2264-2268 and Altschul (1993), *J Mol Evol* 36:290-300, fully incorporated by reference) which are tailored for sequence similarity searching. The approach used by the **BLAST** program is first to consider similar segments between a query sequence and a database sequence, then to evaluate the statistical significance of all matches that are identified and finally to summarize only those matches which satisfy a preselected threshold of significance. For a discussion of basic issues in similarity searching of sequence databases, see Altschul *et al.* (1994), *Nat Genet* 6:119-129) which is fully incorporated by reference. The search parameters for histogram, descriptions, alignments, expect (*i.e.*, the statistical significance threshold for reporting matches against database sequences), cutoff, matrix and filter are at the default settings. The default scoring matrix used by blastp, blastx, tblastn, and tblastx is the BLOSUM62 matrix (Henikoff *et al.* (1992), *Proc Natl Acad Sci USA* 89:10915-10919, fully incorporated by reference). Four blastn parameters were adjusted as follows: Q=10 (gap creation penalty); R=10 (gap extension penalty); wink=1 (generates word hits at every winkth position along the query); and gapw=16 (sets the window width within which gapped alignments are generated). The equivalent Blastp parameter settings were Q=9; R=2; wink=1; and gapw=32. A Bestfit comparison between sequences, available in the GCG package version 10.0, uses DNA parameters GAP=50 (gap creation penalty) and LEN=3 (gap extension penalty) and the equivalent settings in protein comparisons are GAP=8 and LEN=2.

[0035] As used herein a "probe" or "oligonucleotide probe" is defined as a nucleic acid, capable of binding to a nucleic acid sample or complementary control gene nucleic acid through one or more types of chemical bonds, usually through complementary base pairing, usually through hydrogen bond formation. As used herein, a probe may include natural (*i.e.*, A, G, U, C or T) or modified bases (7-deazaguanosine, inosine, *etc.*). In addition, the bases in probes may be joined by a linkage other than a phosphodiester bond, so long as it does not interfere with hybridization. Thus, probes may be peptide nucleic acids in which the constituent bases are joined by peptide bonds rather than phosphodiester linkages.

[0036] Probe arrays may contain at least two or more oligonucleotides that are complementary to or hybridize to one or more of the control genes described herein. Such arrays may also contain oligonucleotides that are complementary or hybridize to at least about 2, 3, 5, 7, 10, 50, 100 or more the genes described herein. Any solid surface to

which oligonucleotides or nucleic acid sample can be bound, either directly or indirectly, either covalently or non-covalently, can be used. For example, solid supports for various hybridization assay formats can be filters, polyvinyl chloride dishes, silicon or glass based chips, *etc.* Glass-based solid supports, for example, are widely available, as well as associated hybridization protocols. (see, *e.g.,* Beattie, WO 95/11755).

[0037] A preferred solid support is a high density array or DNA chip. This contains an oligonucleotide probe of a particular nucleotide sequence at a particular location on the array. Each particular location may contain more than one molecule of the probe, but each molecule within the particular location has an identical sequence. Such particular locations are termed features. There may be, for example, 2, 10, 100, 1000, 10,000, 100,000, 400,000, 1,000,000 or more such features on a single solid support. The solid support, or more specifically, the area wherein the probes are attached, may be on the order of a square centimeter.

1. *Dot Blots*

[0038] The control genes listed in Table 1 and methods of the present invention may be utilized in numerous hybridization formats such as dot blots, dipstick, branched DNA sandwich and ELISA assays. Dot blot hybridization assays provide a convenient and efficient method of rapidly analyzing nucleic acid samples in a sensitive manner. Dot blots are generally as sensitive as enzyme-linked immunoassays. Dot blot hybridization analyses are well known in the art and detailed methods of conducting and optimizing these assays are detailed in U.S. Patent Nos. 6,130,042 and 6,129,828, and Tkatchenko *et al.* (2000), *Biochimica et Biophysica Acta* 1500:17-30. Specifically, a labeled or unlabeled nucleic acid sample is denatured, bound to a membrane (*i.e.,* nitrocellulose) and then contacted with unlabeled or labeled oligonucleotide probes. Buffer and temperature conditions can be adjusted to vary the degree of identity between the oligonucleotide probes and nucleic acid sample necessary for hybridization.

[0039] Several modifications of the basic Dot blot hybridization format have been devised. For example, Reverse Dot blot analyses employ the same strategy as the Dot blot method, except that the oligonucleotide probes are bound to the membrane and the nucleic acid sample is applied and hybridized to the bound probes. Similarly, the Dot blot hybridization format can be modified to include formats where either the nucleic acid

sample or the oligonucleotide probe is applied to microtiter plates, microbeads or other solid substrates.

2. *Membrane-Based Formats*

[0040] Although each membrane-based format is essentially a variation of the Dot blot hybridization format, several types of these formats are preferred. Specifically, the methods of the present invention may be used in Northern and Southern blot hybridization assays. Although the methods of the present invention are generally used in quantitative nucleic acid hybridization assays, these methods may be used in qualitative or semi-quantitative assays such as Southern blots, in order to facilitate comparison of blots. Southern blot hybridization, for example, involves cleavage of either genomic or cDNA with restriction endonucleases followed by separation of the resultant fragments on a polyacrylamide or agarose gel and transfer of the nucleic acid fragments to a membrane filter. Labeled oligonucleotide probes are then hybridized to the membrane-bound nucleic acid fragments. In addition, intact cDNA molecules may also be used, separated by electrophoresis, transferred to a membrane and analyzed by hybridization to labeled probes. Northern analyses, similarly, are conducted on nucleic acids, either intact or fragmented, that are bound to a membrane. The nucleic acids in Northern analyses, however, are generally RNA.

3. *Arrays*

[0041] Any microarray platform or technology may be used to produce gene expression data that may be normalized with the control genes and methods of the invention. Oligonucleotide probe arrays can be made and used according to any techniques known in the art (see for example, Lockhart *et al.*, (1996), *Nat Biotechnol* 14:1675-1680; McGall *et al.* (1996), *Proc Natl Acad Sci USA* 93:13555-13460). Such probe arrays may contain at least one or more oligonucleotides that are complementary to or hybridize to one or more of the nucleic acids of the nucleic acid sample and/or the control genes of Tables 1-3. Such arrays may also contain oligonucleotides that are complementary or hybridize to at least 2, 3, 5, 7, 10, 25, 50, 100, 500 or more of the control genes listed in Tables 1-3.

[0042] Control oligonucleotide probes of the invention are preferably of sufficient length to specifically hybridize only to appropriate, complementary genes or transcripts. Typically the oligonucleotide probes will be at least about 10, 12, 14, 16, 18, 20 or 25 nucleotides in length. In some cases longer probes of at least 30, 40, or 50 nucleotides

will be desirable. The oligonucleotide probes of high density array chips include oligonucleotides that range from about 5 to about 45 or 5 to about 500 nucleotides, more preferably from about 10 to about 40 nucleotides and most preferably from about 15 to about 40 nucleotides in length. In other particularly preferred embodiments, the probes are 20 or 25 nucleotides in length. In another preferred embodiment, probes are double- or single-stranded DNA sequences. The oligonucleotide probes are capable of specifically hybridizing to the control gene nucleic acids in a sample.

[0043] One of skill in the art will appreciate that an enormous number of array designs comprising control probes of the invention are suitable for the practice of this invention. The high density array will typically include a number of probes that specifically hybridize to each control gene nucleic acid, *e.g.* mRNA or cRNA. (See WO 99/32660 for methods of producing probes for a given gene or genes). Assays and methods comprising control probes of the invention may utilize available formats to simultaneously screen at least about 100, preferably about 1000, more preferably about 10,000 and most preferably about 500,000 or 1,000,000 different nucleic acid hybridizations.

[0044] The methods and control genes of this invention may also be used to normalize gene expression data produced using commercially available oligonucleotide arrays that contain or are modified to contain control gene probes or the invention. A preferred oligonucleotide array may be selected from the Affymetrix, Inc. GeneChip® series of arrays which include the Human Genome Focus Array, Human Genome U133 Set, Human Genome U95 Set, HuGeneFL Array, Human Cancer Array, HuSNP Mapping Array, GenFlex Tag Array, p53 Assay Array, CYP450 Assay Array, Rat Genome U34 Set, Rat Neurobiology U34 Array, Rat Toxicology U34 Array, Murine Genome U74v2 Set, Murine 11K Set, Yeast Genome S98 Array, *E. coli* Antisense Genome Array, *E. coli* Genome Array (Sense), *Arabidopsis* ATH1 Genome Array, *Arabidopsis* Genome Array, *Drosophila* Genome Array, *C. elegans* Genome Array, *P. aeruginosa* Genome Array and *B. subtilis* Genome Array. In another embodiment, an oligonucleotide array may be selected from the Motorola Life Sciences and Amersham Pharmaceuticals CodeLink™ Bioarray System microarrays, including the UniSet Human 20K I, Uniset Human I, ADME-Rat, UniSet Rat I and UniSet Mouse I, or from the Motorola Life Sciences eSensor™ series of microarrays.

4. *RT-PCR*

[0045] The control genes and methods of the invention may be used in any type of polymerase chain reaction. A preferred PCR format is reverse transciptase polymerase chain reaction (RT-PCR), an *in vitro* method for enzymatically amplifying defined sequences of RNA (Rappolee *et al.* (1988), *Science* 241:708-712) permitting the analysis of different samples from as little as one cell in the same experiment (See Ambion: RT-PCR: The Basics; M.J. McPherson and S.G. Møller, PCR BIOS Scientific Publishers Ltd., Oxford, OX4 1RE, 2000; Dieffenbach *et al.*, PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1995, for review). One of ordinary skill in the art may appreciate the enormous number of variations in RT-PCR platforms that are suitable for the practice of the invention, including complex variations aimed at increasing sensitivity such as semi-nested (Wasserman *et al.* (1999), *Mol Diag* 4:21-28), nested (Israeli *et al.* (1994), *Cancer Res* 54:6303-6310; Soeth *et al.* (1996), *Int J Cancer* 69:278-282), and even three-step nested (Funaki *et al.* (1997), *Life Sci* 60:643-652; Funaki *et al.* (1998), *Brit J Cancer* 77:1327-1332).

[0046] In one embodiment of the invention, separate enzymes are used for reverse transcription and PCR amplification. Two commonly used reverse transcriptases, for example, are avian myeloblastosis virus and Moloney murine leukaemia virus. For amplification, a number of thermostable DNA-dependent DNA polymerases are currently available, although they differ in processivity, fidelity, thermal stability and ability to read modified triphosphates such as deoxyuridine and deoxyinosine in the template strand (Adams *et al.* (1994), *Bioorg Med Chem* 2:659-667; Perler *et al.* (1996), *Adv Prot Chem* 48:377-435). The most commonly used enzyme, Taq DNA polymerase, has a 5'-3' nuclease activity but lacks a 3'-5' proofreading exonuclease activity. When fidelity is required, proofreading exonucleases such as Vent and Deep Vent (New England Biolabs) or Pfu (Stratagene) may be used (Cline *et al.* (1996), *Nuc Acids Res* 24:3456-3551). In another embodiment of the invention, a single enzyme approach may be used involving a DNA polymerase with intrinsic reverse transcriptase activity, such as *Thermus thermophilus* (Tth) polymerase (Bustin (2000), *J Mol Endo* 25:169-193). A skilled artisan may appreciate the variety of enzymes available for use in the present invention.

[0047] The methodologies and control gene primers of the present invention may be used, for example, in any kinetic RT-PCR methodology, including those that combine fluorescence techniques with instrumentation capable of combining amplification, detection and quantification (Orlando *et al.* (1998), *Clin Chem Lab Med* 36:255-269). The

choice of instrumentation is particularly important in multiplex RT-PCR, wherein multiple primer sets are used to amplify multiple specific targets simultaneously. This requires simultaneous detection of multiple fluorescent dyes. Accurate quantitation while maintaining a broad dynamic range of sensitivity across mRNA levels is the focus of upcoming technologies, any of which are applicable for use in the present invention. Preferred instrumentation may be selected from the ABI Prism 7700 (Perkin-Elmer-Applied Biosystems), the Lightcycler (Roche Molecular Biochemicals) and iCycler Thermal Cycler. Featured aspects of these products include high-throughput capacities or unique photodetection devices.

[0048] Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, practice the methods and use the control genes of the present invention. The following examples therefore, specifically point out the preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

EXAMPLES

Example 1: Selection Of Control Genes

[0049] The control genes were selected by querying a Gene Logic rat tissue database to create expression profiles from a variety of rat cell and tissue samples.

[0050] This database was produced from data derived from screening various cell or tissue samples using the Affymetrix rat GeneChip® set. The rat cell and tissue samples that were analyzed include those that were not treated at all and can be referred to as "normal," as they represent the laboratory rat population that has not been manipulated outside of normal daily activity within that setting. In general, tissue and cell samples were processed following the Affymetrix GeneChip® Expression Analysis Manual. Frozen cells were ground to a powder using a Spex Certiprep 6800 Freezer Mill. Total RNA was extracted with Trizol (GibcoBRL) utilizing the manufacturer's protocol. The total RNA yield for each sample was 200-500 µg per 300 mg cells. mRNA was isolated using the Oligotex mRNA Midi kit (Qiagen) followed by ethanol precipitation. Double stranded cDNA was generated from mRNA using the SuperScript Choice system (GibcoBRL). First strand cDNA synthesis was primed with a T7-(dT24) oligonucleotide. The cDNA was phenol-chloroform extracted and ethanol precipitated to a final concentration of 1

µg/ml. From 2 µg of cDNA, cRNA was synthesized using Ambion's T7 MegaScript in vitro Transcription Kit.

[0051] To biotin label the cRNA, nucleotides Bio-11-CTP and Bio-16-UTP (Enzo Diagnostics) were added to the reaction. Following a 37°C incubation for six hours, impurities were removed from the labeled cRNA following the RNeasy Mini kit protocol (Qiagen). cRNA was fragmented (fragmentation buffer consisting of 200 mM Tris-acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc) for thirty-five minutes at 94°C. Following the Affymetrix protocol, 55 µg of fragmented cRNA was hybridized on the Affymetrix rat array set for twenty-four hours at 60 rpm in a 45°C hybridization oven. The chips were washed and stained with Streptavidin Phycoerythrin (SAPE) (Molecular Probes) in Affymetrix fluidics stations. To amplify staining, SAPE solution was added twice with an anti-streptavidin biotinylated antibody (Vector Laboratories) staining step in between. Hybridization to the probe arrays was detected by fluorometric scanning (Hewlett Packard Gene Array Scanner). Data was analyzed using Affymetrix GeneChip® version 3.0 and Expression Data Mining Tool (EDMT) software (version 1.0), S-Plus, and the GeneExpress® software system. Microarrays were scanned on a high photomultiplier tube (PMT) settings.

[0052] To prepare tissue samples from animals, e.g. rats, sterile instruments were used to sacrifice the animals, and fresh and sterile disposable instruments were used to collect tissues. Gloves were worn at all times when handling tissues or vials. All tissues were collected and frozen within approximately 5 minutes of the animal's death. The liver sections and kidneys were frozen within approximately 3-5 minutes of the animal's death. The time of euthanasia, an interim time point at freezing of liver sections and kidneys, and time at completion of necropsy were recorded. Tissues were stored at approximately - 80°C or preserved in 10% neutral buffered formalin.

[0053] Tissues were collected and processed as follows.

[0054] Liver

    1. Right medial lobe – snap frozen in liquid nitrogen and stored at ~-80°C.

    2. Left medial lobe - Preserved in 10% neutral-buffered formalin (NBF) and evaluated for gross and microscopic pathology.

    3. Left lateral lobe – snap frozen in liquid nitrogen and stored at ~-80°C.

[0055] <u>Heart</u>- A sagittal cross-section containing portions of the two atria and of the two ventricles was preserved in 10% NBF. The remaining heart was frozen in liquid nitrogen and stored at ~ -80°C.

[0056] <u>Kidneys</u> (both)

    1. Left – Hemi-dissected; half was preserved in 10% NBF and the remaining half was frozen in liquid nitrogen and stored at ~ -80°C.

    2. Right – Hemi-dissected; half was preserved in 10% NBF and the remaining half was frozen in liquid nitrogen and stored at ~ -80°C.

[0057] <u>Testes</u> (both)- A sagittal cross-section of each testis was preserved in 10% NBF. The remaining testes were frozen together in liquid nitrogen and stored at ~-80°C.

[0058] <u>Brain</u> (whole)- A cross-section of the cerebral hemispheres and of the diencephalon was preserved in 10% NBF, and the rest of the brain was frozen in liquid nitrogen and stored at ~ -80°C.

[0059] Gene expression data were then analyzed to identify those genes that were consistently expressed across a set of about 5,000 different tissue samples. Table 1 provides a list of approximately 128 genes whose expression, as determined by ANOVA , is considered not to vary across the normal and treated samples studied. Table 1 also provides a GenBank Accession number (fragment name), present frequency and mean average differential for each of the genes. The GenBank Accession Nos. can be used to locate the publicly available sequences, each of which is herein incorporated by reference as of the priority date of this application (July 17, 2002).

[0060] A two-factor ANOVA model was applied to all cell and tissues samples where both control and disease, pathology or treatment groups existed. The factors for this model were normal state (control or affected tissue) and cell or tissue type. A one factor ANOVA was also used to examine the effects of tissue kind alone. Genes were ranked according to R-squared values. The R-squared value can be interpreted as the percent variability of expression that can be explained by the underlying factors. Cut-off values were also selected for the alpha error p-values for each factor and the interaction of these two factors. A cut-off value for both one factor and two factor R-squared values of less than or equal to 12 was used. In addition, any gene with large known regulation events

within tissues was removed and any co-clustered Unigene fragments were examined for consistency in R-Squared values. The probe set was also selected using the following supplemental criteria: (a) Mean Average Differential over all rat samples less than or equal to about 20, (b) Present Frequency over all rat samples less than or equal to about 75% and (c) no probe sets exhibiting saturation.

Model 1: $E_{ij} = u + T_j + error$

(E$_{ij}$ is the expression value of the ith gene in the jth sample)

(T$_j$ is the tissue type of the jth sample)

[0061] The model fitting yields, for each gene, a p-value for the T factor, as well as a sum of squares attributable to this factor. This sum of squares is the model sum of squares. The R^2 value is then the ratio of the model sum of squares to the total sum of squares

$$\sum_j (E_{ij} - \bar{E}_i)^2.$$

Model 2: $E_{ij} = u + T_j + N_j + T_j*N_j + error$

(E$_{ij}$ is the expression value of the ith gene in the jth sample)

(T$_j$ is the tissue type of the jth sample)

(N$_j$ is the state of the jth sample (N$_j$ =0 for normal, 1 otherwise))

[0062] The model fitting yields, for each gene, a p-value for the T factor, the N factor, and the T*N factor, as well as a sum of squares attributable to each of these factors. Adding the three sums of squares gives the model sum of squares. The R^2 value is then the ratio of the model sum of squares to the total sum of squares

$$\sum_j (E_{ij} - \bar{E}_i)^2.$$

[0063] TABLE 1

GLGC Identifier	Fragment Name	Present Frequency	Mean Average Differential
102271	AA012709_at	0.9282	190.551
77300	AF029357cds_at	0.9848	119.409
77332	AF034900mRNA_i_at	0.989	203.019
77517	AF081148_s_at	0.9146	52.382
77576	AF091561_at	0.9609	62.252
77615	AF095927_at	0.9521	40.406
77721	AJ132230_g_at	0.7605	62.179
77738	D01046_at	0.8189	70.892
77745	D10587_at	0.8261	103.633
80151	D87840_at	0.9734	83.52
78209	M13100cds#1_g_at	0.9657	192.653
78211	M13100cds#3_f_at	0.9867	265.171
78212	M13100cds#4_f_at	0.9918	128.404
78213	M13100cds#5_s_at	0.9717	179.794
78214	M13100cds#6_f_at	0.9817	338.825
78215	M13101cds_f_at	0.9256	195.555
81802	M25584_at	0.7688	108.344
76571	M27467_at	0.8166	64.614
76597	M74439mRNA_i_at	0.9709	85.002
76604	M76767_s_at	0.9227	148.154
81918	M83680_at	0.9692	151.235
84412	rc_AA799406_at	0.9722	150.886
84486	rc_AA799551_g_at	0.7849	110.294
84567	rc_AA799745_at	0.8588	123.746
84748	rc_AA800684_at	0.8148	47.537
84809	rc_AA800881_at	0.8955	98.88
84830	rc_AA801017_at	0.8557	56.038
84832	rc_AA801025_g_at	0.9197	88.845
84841	rc_AA801181_at	0.8566	101.242
84851	rc_AA801228_g_at	0.9251	113.4
84854	rc_AA801231_at	0.8871	222.933
99702	rc_AA818590_at	0.7573	32.931
98583	rc_AA819268_at	0.9357	347.913
100600	rc_AA819664_at	0.9852	320.9
84964	rc_AA848965_at	0.8342	64.375
85024	rc_AA849525_i_at	0.8484	45.264
85060	rc_AA849730_at	0.8953	66.225
85158	rc_AA850117_at	0.9611	228.531
85262	rc_AA850595_at	0.9132	86.758
85466	rc_AA851405_at	0.9773	114.684
85474	rc_AA851439_at	0.962	229.271
85553	rc_AA851892_at	0.9836	218.25
102013	rc_AA858480_at	0.8612	110.441
101949	rc_AA859201_at	0.9978	275.683
81000	rc_AA859702_at	0.8713	26.883
83140	rc_AA859750_at	0.7544	51.105
83979	rc_AA892504_at	0.82	109.04

GLGC Identifier	Fragment Name	Present Frequency	Mean Average Differential
81044	rc_AA892895_r_at	0.9972	499.824
84111	rc_AA892959_at	0.8275	37.656
84145	rc_AA893127_at	0.7778	96.525
84310	rc_AA893980_at	0.8572	69.74
84392	rc_AA894340_at	0.8296	31.49
85633	rc_AA899265_at	0.8552	56.148
85635	rc_AA899278_at	0.8469	56.079
85698	rc_AA899664_at	0.9944	414.896
85712	rc_AA899723_at	0.9147	112.458
85771	rc_AA899991_at	0.8249	124.576
85831	rc_AA900348_s_at	0.9502	212.75
85846	rc_AA900422_at	0.9604	404.271
85949	rc_AA900926_at	0.8398	71.065
86913	rc_AA901272_f_at	0.7765	48.604
87063	rc_AA924396_at	0.9271	83.43
76263	rc_AA924542_s_at	0.9604	62.91
87182	rc_AA924830_at	0.7985	40.337
87211	rc_AA924964_at	0.794	393.025
87348	rc_AA925432_at	0.9735	225.799
87443	rc_AA925854_at	0.8516	92.302
86025	rc_AA942964_at	0.9328	494.302
86074	rc_AA943120_at	0.855	233.325
86169	rc_AA943553_g_at	0.9966	665.561
86209	rc_AA943738_g_at	0.9859	137.092
86243	rc_AA943835_at	0.7664	165.778
86314	rc_AA944239_at	0.949	216.561
86524	rc_AA945099_g_at	0.8554	54.104
86629	rc_AA945805_at	0.8566	68.783
86724	rc_AA946166_at	0.9215	75.825
86727	rc_AA946181_at	0.8695	169.878
86837	rc_AA946499_at	0.8446	63.922
86846	rc_AA946528_at	0.9054	279.156
87736	rc_AA955911_at	0.7623	70.604
87993	rc_AA957063_at	0.9941	391.775
88267	rc_AA963170_at	0.987	118.572
88591	rc_AA964611_at	0.9243	128.413
88723	rc_AA965110_at	0.7869	67.276
88766	rc_AA996405_at	0.8167	72.635
88839	rc_AA996701_f_at	0.7552	43.716
89007	rc_AA997745_at	0.7736	45.566
89217	rc_AA997960_at	0.8546	77.485
89360	rc_AA998471_i_at	0.9129	284.784
89468	rc_AA999041_at	0.9482	133.563
89701	rc_AI008674_at	0.8997	100.377
76186	rc_AI009141_at	0.811	67.18
90399	rc_AI011949_at	0.7884	74.517
90427	rc_AI012073_at	0.7986	34.14
90437	rc_AI012103_at	0.7764	479.806

GLGC Identifier	Fragment Name	Present Frequency	Mean Average Differential
90744	rc_AI013204_at	0.9984	974.703
90764	rc_AI013310_at	0.7918	76.764
81319	rc_AI014135_g_at	0.8066	111.16
91024	rc_AI029274_at	0.8263	59.624
81335	rc_AI029805_at	0.8404	27.604
91371	rc_AI030564_at	0.7837	286.222
91449	rc_AI030813_at	0.7509	52.319
91867	rc_AI044239_i_at	0.8506	43.725
92024	rc_AI044638_at	0.9104	212.046
92444	rc_AI045686_at	0.7798	72.274
92887	rc_AI059209_at	0.775	148.062
92926	rc_AI059305_at	0.9861	219.211
93077	rc_AI059664_at	0.9072	154.307
93103	rc_AI059728_f_at	0.8303	281.846
93147	rc_AI059883_at	0.8219	61.436
93198	rc_AI060012_at	0.7549	128.285
93390	rc_AI069980_at	0.7936	325.454
93698	rc_AI070712_at	0.9272	121.653
93822	rc_AI071114_at	0.9722	94.206
93870	rc_AI071210_at	0.8462	85.695
93887	rc_AI071243_at	0.9775	164.564
93927	rc_AI071332_at	0.8399	160.424
93955	rc_AI071418_at	0.7542	35.773
94022	rc_AI071563_at	0.7516	42.418
94095	rc_AI071696_f_at	0.8824	255.85
94127	rc_AI071763_at	0.7685	27.537
94183	rc_AI071902_at	0.8004	29.416
93354	rc_AI071920_at	0.8101	41.866
94624	rc_AI073001_at	0.7888	46.337
94667	rc_AI073105_at	0.8006	41.572
94674	rc_AI073118_at	0.9816	132.82
94690	rc_AI073191_at	0.9111	51.687
96075	rc_AI101659_at	0.9988	627.052
96344	rc_AI102991_at	0.998	389.649
96381	rc_AI103202_at	0.8064	149.589
96436	rc_AI103415_at	0.8165	44.836
94805	rc_AI111950_at	0.941	117.798
81430	rc_AI112391_s_at	0.9029	56.828
95309	rc_AI144587_at	0.8708	39.214
95480	rc_AI145609_at	0.9806	84.399
81469	rc_AI146195_at	0.8938	51.357
95868	rc_AI169293_at	0.9127	64.184
96814	rc_AI169595_at	0.9206	124.878
96999	rc_AI170628_at	0.8098	39.401
97024	rc_AI170715_at	0.7835	50.309
97099	rc_AI170992_at	0.8404	82.011
97125	rc_AI171172_i_at	0.9942	137.021
97394	rc_AI172069_at	0.9579	55.272

GLGC Identifier	Fragment Name	Present Frequency	Mean Average Differential
97458	rc_AI172218_at	0.9678	136.643
97601	rc_AI172576_at	0.8256	38.281
97690	rc_AI175266_at	0.9973	335.31
97837	rc_AI175830_at	0.7816	27.925
97962	rc_AI176309_at	0.9542	86.007
98068	rc_AI176625_at	0.8551	152.373
98219	rc_AI177089_at	0.7707	28.18
98232	rc_AI177117_at	0.7661	54.616
98277	rc_AI177251_at	0.8129	49.094
98367	rc_AI177595_at	0.8043	52.792
98370	rc_AI177603_at	0.798	37.734
98563	rc_AI178446_at	0.8241	98.564
98796	rc_AI179239_at	0.992	158.966
98850	rc_AI179411_at	0.9052	78.786
99019	rc_AI180081_at	0.9738	389.838
99327	rc_AI228249_at	0.9917	429.5
99339	rc_AI228279_at	0.8721	81.722
99439	rc_AI228722_at	0.8644	49.792
99810	rc_AI230308_at	0.9803	180.54
99878	rc_AI230562_at	0.9277	84.362
81702	rc_AI230572_at	0.8913	58.278
100117	rc_AI231330_at	0.751	40.863
100183	rc_AI231565_at	0.9039	104.091
100394	rc_AI232347_at	0.8852	120.621
100501	rc_AI232722_at	0.8026	180.831
100698	rc_AI233529_f_at	0.8144	72.074
100818	rc_AI233965_at	0.9171	60.938
100819	rc_AI233966_at	0.8467	142.163
101057	rc_AI235032_at	0.9552	125.501
101104	rc_AI235232_at	0.8299	102.496
101115	rc_AI235272_at	0.7574	35.891
101135	rc_AI235315_at	0.7708	60.792
101275	rc_AI235821_f_at	0.7721	181.906
101388	rc_AI236169_at	0.9237	82.826
101477	rc_AI236475_at	0.8718	156.175
101721	rc_AI237366_at	0.9603	63.197
80595	rc_AI639114_at	0.8775	21.093
80849	rc_AI639391_at	0.7655	61.047
80925	rc_AI639465_f_at	0.9602	142.244
83528	rc_H31217_at	0.7871	28.269
83544	rc_H31535_at	0.8248	95.236
78445	S50461_s_at	0.7606	35.999
78545	S70803_at	0.884	93.026
78574	S74572_g_at	0.791	32.907
78678	S90449_at	0.8728	27.837
82688	U37138_at	0.8904	47.73
82488	U49099_at	0.9579	89.613
76764	U61184_at	0.8679	32.322

GLGC Identifier	Fragment Name	Present Frequency	Mean Average Differential
78926	U87971_g_at	0.8219	29.276
78969	X05472cds#1_s_at	0.923	129.01
78971	X05472cds#3_f_at	0.8638	129.503
79009	X13527cds_s_at	0.7644	118.765
79081	X53581cds#3_f_at	0.908	166.237
79840	X53944_at	0.9981	196.006
79230	X89697cds_at	0.806	34.392

Example 2: Quantitative PCR Analysis of Expression Levels using the Control Genes

[0064] The expression levels of one or more genes listed in Table 1 may be used to normalize gene expression data produced using quantitative PCR analysis. For example, the sequences may be used as Taqman probes, along with the forward and reverse primers for a gene in Table 1. Real time PCR detection may be accomplished by the use of the ABI PRISM 7700 Sequence Detection System. The 7700 measures the fluorescence intensity of the sample each cycle and is able to detect the presence of specific amplicons within the PCR reaction. The TaqMan® assay provided by Perkin Elmer may be used to assay quantities of RNA. The primers may be designed from each of the genes identified in Table 1 using Primer Express, a program developed by PE to efficiently find primers and probes for specific sequences. These primers may be used in conjunction with SYBR green (Molecular Probes), a nonspecific double-stranded DNA dye, to measure the expression level mRNA corresponding to the expression levels of each gene. This gene expression data may then be used to normalize gene expression data of other test genes.

[0065] Although the present invention has been described in detail with reference to examples above, it is understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited patents and publications referred to in this application are herein incorporated by reference in their entirety.